# A complex of a human FOXC2 protein and a FOXC2-interacting protein

## TECHNICAL FIELD

The present invention relates to complexes of the FOXC2 protein with other proteins, in particular complexes of FOXC2 with proteins designated p621, NOLP, HSC71, FTP3, CLH1, and Kinase A Anchor Protein 84/149 (AKAP). The complexes can be used in methods of identifying agents useful for the treatment of medical conditions which can be treated by modulated FOXC2 activity, such as obesity, hypertriglyceridemia, diet-induced insulin resistance, and/or type 2 diabetes.

## BACKGROUND ART

Obesity, hyperlipidemia, and insulin resistance are common forerunners of type 2 diabetes mellitus. The human winged helix/forkhead transcription factor gene FOXC2 has been identified as a key regulator of adipocyte metabolism (Cederberg, A. et al. (2001) Cell 106:563-573). Increased FOXC2 expression, in adipocytes, has a pleiotropic effect on gene expression, which leads to a lean and insulin sensitive phenotype. FOXC2 affects adipocyte metabolism by increasing the sensitivity of the beta-adrenergic-cAMP-protein kinase A (PKA) signaling pathway through alteration of adipocyte PKA holoenzyme composition. Increased FOXC2 levels, induced by high fat diet, seem to counteract most of the symptoms associated with obesity, including hypertriglyceridemia and diet-induced insulin resistance; a likely consequence hereof would be protection against type 2 diabetes.

The nucleotide and amino acid sequences of the human FOXC2 protein (SEQ ID NO:1), also known as FKHL14, FREAC-11, or S12, as well as the corresponding mouse mesenchyme forkhead-1 (MFH-1) protein, are known in the art, see Miura, N. et al. (1993) FEBS letters 326: 171-176; Miura, N. et al. (1997) Genomics 41: 489-492; WO 98/54216 and WO 01/60853.

Various mechanisms have been proposed for how FOXC2 function to regulate gene expression. One possibility is that FOXC2 interact with factors that are downstream of the Notch-Delta signaling pathway (Kume, T. et al. (2001) Genes & Development 15:2470-2482). For example, Groucho proteins form transcription repression complexes with bHLH transcriptions factors. It has been shown that Groucho can bind to two FOX proteins, FOXG1 and FOXA2 (Wang, J.-C. et al. (2001) J. Biol. Chem. 275: 18418-18423; Yao, J. et al. (2001) Mol. Cell. Biol. 21:1962-1972), and it

was suggested that similar kinds of interactions may occur with FOXC proteins (Kume et al., *supra*). However, an interaction between the FOXC2 protein and Groucho has not previously been demonstrated. Further, interactions of FOXC2 with any of the proteins designated p621, NOLP, Heat Shock Cognate Protein-71 (HSC71), FTP3, CLH1, or Kinase A Anchor Protein 84/149 (AKAP) have not been previously described.

## DISCLOSURE OF THE INVENTION

The present invention is based upon the identification of proteins that interact with FOXC2. The identification of FOXC2-interacting proteins contributes to the understanding of this transcription factor-signaling pathway. Further, such interacting proteins can themselves be useful for the identification of agents useful for the treatment of obesity and diabetes.

Consequently, in a first aspect this invention provides a protein complex of a FOXC2 protein, e.g., a human FOXC2 protein, and a FOXC2-interacting protein, wherein the FOXC2-interacting protein contains an amino acid sequence selected from the group consisting of p621 (e.g., SEQ ID NO:2), NOLP (e.g., SEQ ID NO:3), Heat Shock Cognate Protein-71 (HSC71; e.g., SEQ ID NO:4), FTP3 (e.g., SEQ ID NO:5), CLH1 (e.g., SEQ ID NO:6), and Kinase A Anchor Protein 84/149 (AKAP; e.g., SEQ ID NO:7).

In one embodiment, the invention features a substantially pure protein complex comprising a first polypeptide and a second polypeptide, wherein the first polypeptide comprises the amino acid sequence of SEQ ID NO:1 and the second polypeptide comprises the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7.

In some embodiments the first polypeptide comprises a sequence that differs from the amino acid sequence of SEQ ID NO:1 at one or more residues. In addition, in some embodiments the second polypeptide comprises a sequence that differs from the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7 at one or more residues. The differences are, preferably, differences or changes at a non-essential residue or a conservative substitution. In one embodiment, the first or second polypeptide includes an amino acid sequence at least about 60% identical to a sequence shown as SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, or a fragment thereof. Preferably, the polypeptide is at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more identical to SEQ ID NO:1, 2, 3, 4, 5, 6, or 7. Preferred polypeptide fragments are at least 10%, preferably at least

20%, 30%, 40%, 50%, 60%, 70%, or more, of the length of the sequence shown as SEQ ID NO:1, 2, 3, 4, 5, 6, or 7. The first polypeptide preferably retains the ability to bind to the polypeptide of any of SEQ ID NO:2, 3, 4, 5, 6, or 7. In some examples, the first polypeptide has FOXC2 transcriptional activity. The second polypeptide preferably retains the ability to bind to the polypeptide of SEQ ID NO:1.

The term "substantially pure" as used herein in reference to a given protein complex or polypeptide means that the protein complex or polypeptide is substantially free from other biological macromolecules. For example, the substantially pure protein complex or polypeptide is at least 25%, 50, 75, 80, 85, 95, or 99% pure by dry weight. Purity can be measured by any appropriate standard method known in the art, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

In another aspect, the invention provides a pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a complex of a human FOXC2 protein and a FOXC2-interacting protein, wherein the FOXC2-interacting protein has an amino acid sequence chosen from the group consisting of SEQ ID NO:2, 3, 4, 5, 6, 7 or 8, and a pharmaceutically acceptable carrier.

Another embodiment of the invention is a pharmaceutical composition for use in the treatment of a medical condition which is treatable by modulated FOXC2 activity, comprising a therapeutically or prophylactically effective amount of a FOXC2-interacting protein having an amino acid sequence chosen from the group consisting of SEQ ID NO:2, 3, 4, 5, 6, 7 or 8, and a pharmaceutically acceptable carrier.

Another embodiment of the invention is a pharmaceutical composition comprising an amount of a protein complex described herein effective for the treatment or prevention of a medical condition associated with FOXC2 expression or activity, and a pharmaceutically acceptable carrier.

Yet another aspect of the invention is a method of modulating FOXC2 expression or activity, the method comprising contacting a cell expressing FOXC2, e.g., human FOXC2, with an amount of a polypeptide, e.g., a substantially pure polypeptide, described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7), or a nucleic acid encoding the polypeptide, sufficient to modulate the expression or activity of FOXC2 in the cell.

A further aspect of the invention is a method for the treatment or prophylaxis of a medical condition treatable by modulated FOXC2 activity, the method comprising

administering to a patient in need of such treatment or prophylaxis an amount of a polypeptide, e.g., a substantially pure polypeptide, described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7) effective to treat or prevent a medical condition treatable by modulated FOXC2 activity, and a pharmaceutically acceptable carrier

The medical condition can be a medical condition that is putatively treatable by increased FOXC2 activity, such as obesity, hypertriglyceridemia, diet-induced insulin resistance, or type 2 diabetes. Alternatively, the medical condition can be a medical condition that is putatively treatable by decreased FOXC2 activity, such as anorexia.

The term "treatment" means any treatment of a diseases in a mammal, including: (i) preventing the disease, i.e. causing the clinical symptoms of the disease not to develop (prophylaxis); (ii) inhibiting the disease, i.e. arresting the development of clinical symptoms; and/or (iii) relieving the disease, i.e. causing the regression of clinical symptoms. The term "effective amount" means a dosage sufficient to provide treatment for the disease state being treated. This will vary depending on the patient, the disease and the treatment being effected.

In another aspect, the invention features a method of identifying an agent that modulates (increases or decreases) the formation of a FOXC2 protein complex, the method comprising: (i) contacting a first polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:1) and a second polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7) in the presence of a candidate agent; (ii) measuring the formation of a complex between the first polypeptide and the second polypeptide in the presence of the candidate agent; and (iii) comparing the formation of the complex between the first polypeptide and the second polypeptide in the presence of the candidate agent with the formation of a complex between the first polypeptide and the second polypeptide in the absence of the candidate agent, to thereby determine whether the candidate agent modulates the formation of a FOXC2 protein complex.

The invention also features a method of identifying an agent that modulates a FOXC2 activity, the method comprising: (i) contacting a first polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:1) and a second polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7) in the presence of a candidate agent; (ii) measuring a FOXC2 activity of the first polypeptide in the presence of the candidate

agent; and (iii) comparing the FOXC2 activity of the first polypeptide in the presence of the candidate agent with the FOXC2 activity of the first polypeptide in the absence of the candidate agent, to thereby determine whether the candidate agent modulates a FOXC2 activity.

The invention also features a method of identifying an agent that modulates a FOXC2 activity, the method comprising: (i) contacting a first polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, or 7) with a candidate agent; (ii) determining that the candidate agent binds to the first polypeptide; (iii) contacting a second polypeptide described herein (e.g., a polypeptide comprising the amino acid sequence of SEQ ID NO:1) with the candidate agent; (iv) measuring a FOXC2 activity of the second polypeptide in the presence of the candidate agent; and (v) comparing the FOXC2 activity of the second polypeptide in the presence of the candidate agent with the FOXC2 activity of the second polypeptide in the absence of the candidate agent, to thereby determine whether the candidate agent modulates a FOXC2 activity.

The first polypeptide and/or the second polypeptide used in the methods can optionally be a substantially pure polypeptide. The methods of the invention can be carried out using a cell-based system or in a cell-free system. An example of a FOXC2 activity that can be measured in the methods of the invention is a FOXC2 transcriptional activity.

The invention also provides a method for the treatment or prophylaxis of a medical condition treatable by modulated FOXC2 activity, the method comprising administering to a patient in need of such treatment or prophylaxis an amount of an agent identified by a method described herein that is effective to treat or prevent a medical condition treatable by modulated FOXC2 activity, and a pharmaceutically acceptable carrier.

The medical condition can be a medical condition that is putatively treatable by increased FOXC2 activity, such as obesity, hypertriglyceridemia, diet-induced insulin resistance, or type 2 diabetes. Alternatively, the medical condition can be a medical condition that is putatively treatable by decreased FOXC2 activity, such as anorexia.

In a further aspect, the invention provides antibodies directed against a complex of a human FOXC2 protein and a FOXC2-interacting protein, which complex is defined above according to the invention. Such antibodies can be prepared according to methods well known in the art. The said antibodies are useful e.g. in methods for the

characterization and/or purification of the human FOXC2 protein and/or a FOXC2-interacting protein wherein a specific binding of the antibody to the said complex are utilized. Such methods can include e.g. immunoprecipitation, immunoblotting, or immunoaffinity chromatography. Immunoprecipitation consists on a multiple ordered steps including cells lysis, binding of a specific antigen to an antibody, precipitation of the antigen-antibody complex, washing and dissociation of the antigen from the immune complex (Current Protocols in Molecular Biology, Chapter 10:Analysis of Proteins, 1991, John Wiley & Sons, Inc.) Immunoblotting is a method that combines the resolution of gel electrophoresis with the specificity of immunochemical detection. Immunoblotting can be used to determine a number of important characteristics of protein antigen (i.e., the presence and quantity on a sample, molecular weight, etc.). It can be combined with immunoprecipitation to allow a very sensitive detection of minor antigens and to study specific interactions between antigens (Antibodies, A Laboratory Manual, Chapter 12: Immunoblotting, 1998, Harlow & Lane, CSH). Immunoaffinity chromatography enables for the purification of soluble or membrane-bound protein antigens from cells or homogenized tissues. The technique involves the elution of a single protein from an immunoaffinity column after prior elution of nonspecific absorbed proteins (Current Protocols in Protein Science, Chapter 9: Affinity purification, 1996, John Wiley & Sons, Inc.).

In one embodiment, the invention features a method for purifying a FOXC2-interacting protein, the method comprising: (i) contacting a protein complex comprising a FOXC2 protein comprising the amino acid sequence of SEQ ID NO:1 and a FOXC2-interacting protein comprising the amino acid sequence of SEQ ID NO:2, 3, 4, 5, 6, 7 or 8 with an antibody that binds to the protein complex; and (ii) purifying the FOXC2-interacting protein from the protein complex. An antibody that binds to the protein complex can also be used to purify the protein complex and/or the FOXC2 protein. Any of the first and/or second polypeptides described herein can be used in the antibody-based purification methods of the invention.

Throughout this description the terms "standard protocols" and "standard procedures", when used in the context of molecular biology techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A

laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Suitable methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Below, the invention is described in the appended examples, which are intended to illustrate the invention, without limiting the scope of protection.

## EXAMPLES

### EXAMPLE 1: Identification of putative positive FOXC2 interacting clones by SRS yeast two-hybrid system

*1.1. Overview of the Sos Recruitment System (SRS)*

The Sos Recruitment System (SRS) was used to assay for polypeptides interacting with the human FOXC2 polypeptide. SRS is a modification of the well-known yeast two-hybrid system first described by Fields & Song (1989) Nature 340, 245-246.

In the CytoTrap® SRS (*http://www.stratagene.com/vectors/signal_trans/cytotrap*; see also Aronheim, A. et al. (1997) Mol. Cell. Biol. 17:3094-3102; and US 5,776,689), proteins are expressed in the cytoplasm where, unlike in the nucleus, they may undergo posttranslational modifications. Protein-protein interactions in the cytoplasm are detected by recruitment of the human Sos gene product (hSos) to the membrane of the cell where it activates the Ras pathway. The CytoTrap system uses the unique yeast strain cdc25H, which contains a temperature-sensitive mutation in the cdc25 gene, the yeast homologue for hSos. This protein, a guanyl nucleotide exchange factor, is essential for activation of the Ras pathway and ultimately for the survival and growth of the cell. The mutation in the cdc25 protein is temperature sensitive; the cells can grow at 25°C but not at 37°C. This

cdc25 mutation can be complemented by the hSos gene product to allow growth at 37°, providing that the hSos protein is localized to the membrane via a protein-protein interaction.

The pMyr vector is designed for cDNA library construction. Genes are expressed in this vector as a fusion protein with the src myristylation signal that targets and anchors the protein to the cell membrane with the gene product extruding into the cytoplasm. Protein expression is controlled by the GAL1 promoter, which is induced in the presence of galactose but repressed in the presence of glucose.

The bait protein (FOXC2) is expressed as a fusion protein with the hSos protein from the pSos vector. When the cDNA library and the bait construct are cotransformed into the cdc25H yeast strain, the only cells capable of growing at 37°C on galactose medium are those that have been rescued by a protein-protein interaction recruiting hSos to the cell membrane.

1.2. Cloning of human FOXC2 in pSos

Human full-length FOXC2 (amino acids 1-501) from the pCB6+ plasmid (Cederberg, A. et al. (2001) Cell 106: 1-20) using CSIX-17 (SEQ ID NO:9) and CSIX-18 (SEQ ID NO:10) primers was amplified by PCR according to standard procedures. The amplified fragment was cloned into the pSos vector (Stratagene catalog No. 217433) using *Bam*HI sites included in the primers. The insert orientation was analyzed by restriction digestion, and the FOXC2 sequence was confirmed by nucleotide sequencing according to standard procedures.

1.3. Expression of pSos-FOXC2 in yeast

The yeast strain cdc25H (Stratagene catalog No. 217437) was transformed with pSos-FOXC2 according to the protocol (Stratagene; CytoTrap Vector Kit; catalog No. 217438) and plated on SD/PDO-Leu plates. Protein extracts were made as described (Moilanen A. et al. (1998) Mol. Cell Biol. 18: 5128-5139) and subsequently analyzed by PAGE and Western blotting using anti-mouse Sos antibodies (BD Transduction Laboratories; catalog No. S15520-050). The western blot analysis showed protein bands migrating with the expected molecular weight corresponding to Sos-FOXC2 (MW 178 kDa) and Sos (MW 127 kDa). Additional bands of lower molecular weight were observed, probably due to protein degradation occurring during extract preparation or in the yeast cells during growth.

1.4 Transformation of yeast cells

A human fetal brain cDNA library (Stratagene catalog No. 975204) was used for transformation of yeast cells. The library was amplified by plating approximately 200,000 colonies/plate of LB-Kan (14-cm diameter). Since the library titer was 0.3 x $10^9$ cfu/ml a total 50 plates inoculated with 0.66 µl of library suspension per plate were used. Cells were incubated overnight at 37°C and afterwards, colonies of a pinpoint size were harvested with 2x4 ml of LB using a sterile scraping glass. Additional LB-Kan medium was added to a final volume of 1;5 l. The cell suspension was incubated for 2 h at 37°C. Cells were harvested by centrifugation at 6,000 x g for 10 min and the plasmid DNA was prepared using Plasmid Maxi Prep columns (Qiagen catalog No. 12162) following the QIAGEN protocol.

Transformation was performed as described by Stratagene (CytoTrap XR Library Construction Kit; Instruction Manual; catalog No. 200444), with the difference that the transformation was sequential, i.e. carried out in two steps. First, yeast was transformed with the pSOS-FOXC2 plasmid. Cdc25H yeast cells carrying the pSos-FOXC2 plasmid were made competent and transformed with 80 µg of cDNA library DNA. After 72 hours of growth at 25°C in glucose (–Leu –Ura) the plates were replicated into a galactose medium and incubated at 37°C for a maximum of 11 days. Transformants were screened following Stratagene protocols for the revertants test.

Approximately 8 x $10^5$ yeast transformants were screened and 4,000 galactose-dependent candidate clones were obtained. After a parallel growth test at non-permissive temperature in glucose and galactose media, 230 of these clones grew only in galactose and were analyzed further. Clones growing in both glucose and galactose media were considered to be revertants and were therefore discarded.

EXAMPLE 2: Analysis of putative positive interacting clones

*2.1. Sequence analysis of putative FOXC2-interacting clones*

Total yeast DNA was prepared as described by Stratagene (CytoTrap XR Library Construction Kit; Instruction Manual; catalog No. 200444). The final pellet was dissolved in 20 µl $H_2O$ and used as template for PCR amplification or transformation of *E. coli* cells. 40 µl TOP10 7' electrocompetent cells were transformed (2.5 kV, 25 µF and 200 Ω) with 2 µl of this DNA. Immediately, 1 ml of SOC medium was added and

cells were incubated for 1 hour at 37°C. All cells were plated onto LB-plates containing 30 μg/ml of chloramphenicol. Transformants were used for plasmid DNA preparations (QIAGEN).

In order to amplify prey inserts the extracted yeast DNA was used as template. The PCR reaction was set up by mixing 1 μl of desired yeast DNA, 1xPCR buffer, 5 units TaqPol, 40 pmol each of the primers NA15 (SEQ ID NO:11) and NA1149 (SEQ ID NO:12) and 200 μM dNTP's to a final volume of 50 μl. The following PCR reaction was started: 95°C for 5 minutes followed by 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 55°C and 1.5 minute at 72°C and a final 7 minutes at 72°C. The fragments obtained were purified and sequenced.

Sequence analysis was performed at the level of PCR or plasmid DNA by BLAST homology search against a non-redundant nucleotide database without ESTs (EMBL and GenBank).

2.2. Identification of seven FOXC2-interacting proteins

Among the expected false positives, several clones encoding hSos, Ras, and other members of Ras-GTPase family were identified, confirming the ras-signaling pathway read-out for this assay. The remaining clones corresponded to previously characterized genes (139 clones) and unknown genes (16 clones). In both cases, some of them were found several times as identical clones, probably due to library amplification. The unknown clones were analyzed further using the validation test in yeast described above. These clones were shown not to express proteins capable of specific interactions with FOXC2, and were therefore disregarded. The clones corresponding to known genes could be classified into the following protein categories: transcription regulators, matrix proteins, transcription factors, kinase-subunits, and nuclear proteins. In total, 43 clones were identified as putative "hits" and further analyzed.

In order to eliminate hits with a nonspecific interaction to FOXC2 (e.g. proteins interacting with the Sos tag-protein) the 43 identified clones were subjected to a false positive test. This was done by co-transformation of cdc25H yeast with each of the hit proteins (pMyrHit) together with (a) pSos-FOXC2; (b) pSos; or (c) as a control, Sos fused to MafB (Stratagene; CytoTrap Vector Kit; catalog No. 217438). Cells that grew in galactose at 37°C only when transformed with plasmid (a) were considered to represent a true positive interaction. By this procedure, seven proteins (Table I) were

identified as putative FOXC2-interacting proteins. For these seven proteins, the above experiment was repeated also with Col1 (Stratagene; CytoTrap Vector Kit; catalog No. 217438) as a control, which gave the same results.

To characterize further the interactions between FOXC2 and FOXC2-interacting proteins in yeast, the interaction of Sos-FOXC2 hybrid protein was compared with the one between MafB proteins. MafB proteins are known to form dimers (Kataoka, K. et al. (1994) Mol. Cell. Biol. 14: 7581-7591). The p621 gene exhibited the strongest interaction to FOXC2 followed by FTP3, Groucho and Clathrin. A weaker interaction was observed for PKA anchor protein, NOLP and HSC71.

TABLE I lists putative FOXC2-interacting proteins identified by SRS. Interaction strength is determined relative to the interaction between MafB proteins (++++) during the same conditions.

TABLE I

| Gene | SEQ ID NO: | Accession No. | Interaction strength |
|---|---|---|---|
| p621 | 2 | AJ242978 (partial mRNA) | ++++ |
| NOLP | 3 | AB017800 | + |
| HSC71 | 4 | BC007276 | + |
| FTP3 | 5 | P55795 | +++ |
| CLH1 | 6 | D21260 | +++ |
| AKAP149 | 7 | X97335 | ++ |
| AES-1/2 / Groucho | 8 | U04241 AAD00654 | +++ |

EXAMPLE 3: Characterization of FOXC2-interacting proteins

*3.1. p621*

p621 (SEQ ID NO:2; partial sequence) is a protein of unknown function that interacts with the Sp1 transcription factor (Gunther, M. et al. (2000) Mol. Cell. Biol. 210: 131-142). The mouse homologue, ATFa-associated factor (mAM), has recently been cloned and characterized (De Grave, F. et al. (2000) 19: 1807-1819). It acts as a

transcriptional co-repressor, and contains a bipartite NLS (Nuclear Localization Signal) and an ATPase activity.

In the present SRS screen, the interaction between FOXC2 and the p621 protein in yeast was supported by 12 obtained clones, comprising three different overlapping sequences. On basis of the identified fragments, the p621 region comprising nucleotides 580-1320 is sufficient for the FOXC2–p621 interaction.

*3.2. NOLP*

NOLP (for "nucleolar-localized protein") is a nucleolar protein cloned from a human fetal brain cDNA library (Ueki, N. et al. (1998) Biochem. Biophys. Res. Comm. 252: 97-102). The NOLP gene encodes a 524-amino acid polypeptide with an *E. coli* helicase-homologous region, an acid-rich domain, three base-rich putative nuclear localization signals, a serine-rich region, and a coiled-coil domain. Northern blot analysis and RT-PCR revealed that NOLP is expressed as a 3.5-kb mRNA in fetal brain, adult brain, and testis. Deletion studies revealed that NOLP contains functional nuclear and nucleolar localization signals. In the present SRS screen, a single NOLP clone was identified, comprising a sequence that starts at the D145 residue of the NOLP sequence (SEQ ID NO:3).

3.3. Heat Shock Cognate Protein-71 (HSC71)

Heat Shock Cognate Protein-71 protein (HSC71; SEQ ID NO:4) has been recently identified from human brain tissues (GenBank Accession No. BC007276). The HSC71 protein contains a hsp70 domain (Pfam-PF00012; Bateman et al. (2002) Nucleic Acids Research 30:276-280) and it is possible to speculate that as other members of this hsp70 superfamily of proteins is involved in protein folding and assembling/disassembling of protein complexes. This has been suggested for the HSC71 protein isolated from rainbow trout (Zafarullah, M. et al. (1992) Eur. J. Biochem. 204: 893-900).

In the present SRS screen, nine HSC71 clones were obtained. They could be categorized into four different overlapping clones (from $K_{67}$ to the stop codon of HSC71 protein).

*3.4. FTP3*

FTP3 (SEQ ID NO:5) is a Heterogeneous Nuclear Ribonucleoprotein-H' (hnRNP-H'), and is ubiquitously expressed. It comprises three RNA-binding motifs and its function may include pre-mRNA processing and transport. hnRNPs are known to bind heterogeneous nuclear RNA, the transcripts produced by RNA polymerase II (Honoré, B. et al. (1995) J. Biol. Chem.270: 28780-28789). In the present SRS screen, a single FTP3 clone was identified, corresponding to the C-terminal region of FTP3 ($D_{348}$-$A_{449}$).

*3.5. CLH1*

CLH1 (SEQ ID NO:6) is a human clathrin heavy chain protein. The clathrin heavy chain is the main structural protein of the cytoplasm surface of coated pits and vesicles, involved in receptor-mediated endocytosis, secretion and intracellular transfer of membrane-associated components. It is located at the cytoplasmic phase of coated pits and vesicles and it is readily expressed in most human adult tissues and localized to human chromosome 17 (Dodge, GR. et al. (1991) Genomics 1:174-178).

In the present SRS screen, 13 similar clones were identified, aligning at the N-terminal to $N_{853}$ amino acid residue of the CLH1 sequence.

3.6. AKAP149 (A Kinase Anchor Protein 149)

The effects of individual protein kinases (PKAs) isoforms are determined by their cellular localization, specified through binding to distinct A Kinase Anchor Proteins (AKAPs). AKAP149 (SEQ ID NO:7; Trendelenburg, G. et al. (1996) Biochem. Biophys. Res. Comm. 225: 313-319) is a putative splicing variant of S-AKAP84 (previously described by Lin et al. (1995) J. Biol. Chem. 270: 27804-27811; GenBank Accession No. U34074) with the important new feature of a RNA-binding motif (KH domain). Trendelenburg et al. showed that AKAP149 was expressed as a 4.2-kb transcript in all epithelial tissues examined, with the strongest signal being detected in prostate and small intestine RNAs. In addition, a 3.2-kb transcript was expressed exclusively in testis. Trendelenburg et al. speculated that AKAP149 is involved in the cAMP-dependent signal transduction pathway and in directing RNA to a specific cellular compartment.

In the present SRS screen, two clones were identified, both containing the entire CDS amino acid sequence of AKAP149.

*3.7. AES1-2/Groucho*

AES1-2/Groucho (SEQ ID NO:8) is a human protein exhibiting approximately 50% identity to the N-terminal region of *Drosophila* "enhancer of split Groucho" protein (Miyasaka, H. et al. (1993) Eur. J. Biochem. 216: 343-352). It is possibly involved in the negative regulation of proteins containing WD40 repeats. It has a nuclear localization and is expressed predominantly in muscle, heart and placenta. In the present SRS screen, two clones were identified.

*3.8. Summary*

In summary, seven FOXC2-interacting proteins were isolated. Two of these proteins (p621 and AES1-2/*groucho*) are involved in transcription and could act by repressing FOXC2 transcriptional activity. In addition, three cytoplasmic proteins (AKAP, Clathrin and HSC71) involved in cellular and matrix localization, and protein folding activity, were identified. Finally, two proteins of nuclear localization; one involved in RNA processing (FTP3) and one of unknown function (NOLP), were identified.

EXAMPLE 4: Expression profiling of FOXC2-interacting proteins

To determine the tissue transcript expression profile for the FOXC2-interacting proteins described in Example 3, a computer analysis of Affymetrix chips containing human transcripts from adipose tissue, liver and muscle was performed.

PolyA+ mRNAs were extracted from human tissues from healthy patients using a Dynabeads mRNA Direct™ kit (Dynal A.S., Norway). White adipose, liver and muscle tissues were from biopsies. mRNAs were reverse transcribed using a T7-tagged oligo-dT primer and double-stranded cDNAs were generated. These cDNAs were then amplified and labeled using in vitro transcription (IVT) with T7 RNA polymerase and biotinylated nucleotides. The populations of cRNAs obtained after IVT were purified and fragmented by heat to produce a distribution of RNA fragment sizes from approximately 35 to 200 bases. The Human Genome U95 Set of five GeneChip® probe arrays (Affymetrix; catalog Nos. 900303, 900305, 900307, 900309 and 900311) were

hybridized using the recommended buffer overnight at 45°C with the denatured cRNA samples. The arrays were then washed and stained with R-phycoerythrin streptavidin with the help of an Affymetrix fluidics station. The cartridges were scanned using a Hewlett-Packard confocal scanner and the images were analyzed with the GeneChip 4.1 software (Affymetrix). The identity of the genes represented on the probe arrays was assessed by performing searches using BLAST (Altschul et al. (1990) J. Mol. Biol. 215: 403-410) on available protein sequence databanks.

The results indicated that all identified FOXC2-interacting proteins are present in adipose and liver tissue, except for NOLP and clathrin proteins. In muscle, all identified FOXC2-interacting proteins are present except for NOLP. It can be concluded that the FOXC2-interacting proteins are expressed in tissues involved in energy metabolism and therefore putatively relevant to medical conditions relating to diabetes and obesity.

EXAMPLE 5: Co-immunoprecipitation

Co-immunoprecipitation of proteins from whole-cell extracts is a valuable approach to test for physical interactions between proteins of interest (Current Protocols in Molecular Biology, Chapter 20: Analysis of protein interactions, 2000, John Wiley & Sons, Inc. 2000). For instance, FOXC2 and each of the identified FOXC2-interacting proteins can be in vitro transcribed/translated under the control of T7 promoter in experiments using a TNT®Coupled Reticulocyte Lysate System (Promega, 2800 Woods Hollow Road, Madison-WI53711, USA) in the presence of $^{35}$S-methionine. The FOXC2-hit complex can be immunoprecipitated using antibodies against FOXC2 or an epitope tag present in one of the proteins expressed as a tag-fusion protein (e.g. c-myc monoclonal or AH-polyclonal antibodies from Clontech). The complex can be resolved by SDS-PAGE. The subsequent exposure of the gel to an X-ray film or phosphorimaging screen can identify the presence of bands of expected size corresponding to the FOXC2-hit complex if these proteins interact to FOXC2.

EXAMPLE 6: Preparation of anti-FOXC2 antibodies

Antibodies are an important tool in the analysis of protein-protein interaction (see e.g. Current Protocols in Molecular Biology, Chapter 11: Immunology, John Wiley & Sons, Inc.). The human FOXC2 protein, or synthetic fragments of the FOXC2 sequence which are specific and antigenic, can be used to immunize animals such as

rabbits. Polyclonal antibodies can be raised following standard protocols (Antibodies, A laboratory Manual, Chapter 5: Immunizations, 1988, Harlow & Lane, CHS) and affinity purified from the whole sera when using peptides as antigen. The antibodies will be useful for co-immunoprecipitation of the FOXC2/FOXC2-interacting protein complex, as well as for western blot analysis of the resolved complex.